

**Capsule deletion via a  $\lambda$ -Red knockout system perturbs biofilm formation and fimbriae expression in *Klebsiella pneumoniae* MGH 78578**

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## Materials and reagents

### Bacterial strains

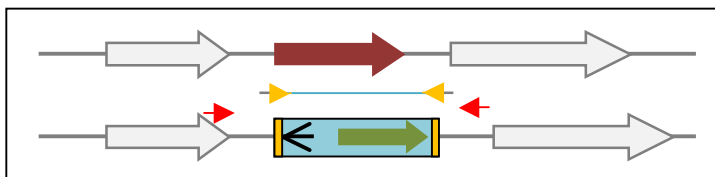
- a) *Klebsiella pneumoniae* MGH 78578

### Plasmids (see Appendix)

- a) pIJ773
- b) pACBSR-Hyg
- c) pFLP-Hyg

### Primers (see Appendix for an example)

- a) Knockout primers (yellow)
- b) Confirmation primers (red)



### Media

- a) Low salt LB broth or agar (per liter)
  - a. Yeast extract: 5 g
  - b. Tryptone: 10 g
  - c. NaCl: 5 g
  - d. Adjust to pH = 8.0 with NaOH
  - e. For plates, Agar: 15 g
  - f. 100 µg/ml hygromycin B (e.g. Invitrogen #10687-010 or Sigma #H3274), as appropriate

Note: Hygromycin must be used with low salt LB, not regular LB
- b) LB broth or agar with 50 µg/ml apramycin (e.g. Sigma #A2024 or RPI #A50020) (LBApra), as appropriate
- c) SOC medium
- d) 1 M sterile filtered L-arabinose. Store at 4°C, and do not use if older than 3 months.
- e) Ice-cold 10% glycerol

### Miscellaneous

- a) Electroporation cuvette 0.2 cm (BioRad #165-2086)
- b) Electroporator (Eppendorf 2510)
- c) PCR reagents (DNA polymerase, nucleotides, etc.)
- d) PCR clean-up kit (e.g. Qiagen QIAquick kit)
- e) DNA gel extraction kit
- f) Sterile inoculating loops
- g) Sterile toothpicks
- h) Gel loading pipette tips (Fisher #02-707-174)

## Timeline

### **Part I. Chromosomal gene replacement**

Day 0. Culture the strain in 3 mL LB at 37°C overnight. Amplify FRT-flanked Apra<sup>R</sup> cassette from pIJ773.

Day 1. Transform with pACBSR-hyg.

Day 2. Culture single colony of *K. pneumoniae* + pACBSR-Hyg in 3 mL low salt LB broth + hygromycin at 30°C overnight.

Day 3. Transform with PCR-amplified knockout cassette.

Day 4. Screen for transformants that have correct insertion of the knockout cassette. Streak correct transformants for single colonies on LBApra at 37°C overnight.

Day 5 - 7. Pick 12 – 15 single colonies of the confirmed mutant and streak onto LBApra plates for 3 days at 37°C.

Day 8. Screen for loss of pACBSR-Hyg by streaking onto LBApra and low salt LB + hygromycin plates at 37°C overnight.

### **Part II. Removal of resistance marker**

Day 9. Culture mutant in 3 mL LBApra at 37°C overnight.

Day 10. Transform with pFLP-hyg.

Day 11. Heat-shock mutants at 43°C overnight.

Day 12. Screen colonies for loss of apramycin resistance.

Day 13 - 15. Pass apramycin-sensitive mutants on LB plates 3 times.

Day 16. Screen for loss of pFLP-hyg by plating on LB and low salt LB + hygromycin plates at 37°C overnight.

Day 17. Confirm gene deletion by PCR and sequencing.

Day 18. Store strain at -80°C.

## Preparing template DNA for amplification of the apramycin resistance gene

- |                     |       |
|---------------------|-------|
| 1. pIJ773           | 5 µg  |
| 2. NEB buffer 2     | 10 µL |
| 3. EcoRI            | 2 µL  |
| 4. HindIII          | 2 µL  |
| 5. H <sub>2</sub> O | 66 µL |

Total volume = 100 µL

Incubate 90 – 120 minutes at 37°C

While the DNA is digesting, prepare a gel (0.8% or 1% agarose) with a well large enough to hold 110 µL.

After incubation, add loading dye, then run on gel (120V for 30 min).

There will be two bands, a very dark one at 3 kb and a much lighter one at 1.3 kb. Using a sharp scalpel, cut out the smaller 1.3 kb fragment and perform a gel extraction. This is the template containing *aac(3)/V* and flanking FRT sites.

## Gene deletion protocol

### Part I. Chromosomal gene replacement

Day 0. A) Culture the strain in 3 mL LB at 37°C overnight.

B) Amplify FRT-flanked Apra<sup>R</sup> cassette from pIJ773.

PCR Conditions			
# Cycles		Temp (°C)	Time (min)
1	Initial activation	95	15
	Denaturation	95	0.5
30	Annealing	55	0.5
	Extension	72	1.5
1	Final Extension	72	10
1	Post-PCR	4	∞

Purify the PCR product using a commercial PCR purification kit.

### Day 1. Transform with pACBSR-hyg.

- 1) Inoculate 1 mL of the overnight culture into 100 mL of fresh LB in a 250 mL Erlenmeyer flask. Incubate at 37°C with shaking or spinning to aerate the culture.

Note: we placed the flask on top of a magnetic stir plate, and a magnetic stir bar inside the flask was spun to aerate the culture. The speed was approximately 800 – 1000 rpm.

- 2) When the OD<sub>600</sub> reaches 0.4 – 0.6 (~2.5 hours), make electrocompetent as follows.

**IMPORTANT: It is very critical to keep everything ICE-COLD!**

- a. Centrifuge the culture for 5 min at 6,500 rpm (7,000 x g).
- b. Carefully decant the LB media and discard.
- c. (Wash #1) Add 50 mL of ice-cold 10% glycerol and resuspend the pellet (*Vortexing at this step is OK*).
- d. Centrifuge again at 6,500 rpm for 5 min. Plunge the centrifuge tube into ice immediately after removal from the centrifuge.
- e. Carefully decant the supernatant and discard.
- f. (Wash #2) Add 50 mL of ice-cold 10% glycerol and resuspend by pipetting up and down. Keep the centrifuge tube in the ice.
- g. Centrifuge again at 6,500 rpm for 5 min.
- h. Carefully decant the supernatant and discard.
- i. (Wash #3) Add 50 mL of ice-cold 10% glycerol and resuspend by pipetting up and down. Keep the centrifuge tube in the ice.

- j. Centrifuge again at 6,500 rpm for 5 min.
  - k. Carefully decant the supernatant and discard.
  - l. Resuspend the pellet in the residual glycerol by pipetting up and down with a 1000  $\mu$ L pipet.
- 3) Electroporate the cells.
- a. Set the voltage on the electroporator to 2,500 V.
  - b. Add 200 – 400 ng of pACBSR-Hyg to a 1.5 mL microfuge tube. To this tube, add 50  $\mu$ L of the resuspended cells. Mix by pipetting up and down.
  - c. Transfer the mixed suspension to a cold electroporation cuvette (0.2 cm gap) using a gel loading tip.
  - d. Tap the cuvette to bring all cells to the bottom.
  - e. Electroporate. (Pulse time is usually around 5-6 seconds).
- 4) Add 250  $\mu$ L of SOC media into the cuvette, and transfer the mixture to a new sterile 1.5-mL microfuge tube using a long gel-loading tip.
- 5) Incubate with gentle shaking for 60 min at **30°C**.
- 6) Spread 100  $\mu$ L of the culture onto a low salt LB + hygromycin plate and incubate at **30°C** overnight.

**Day 2. Culture a single colony of *K. pneumoniae* + pACBSR-Hyg in 3 mL low salt LB broth + hygromycin at 30°C overnight.**

This culture can also be stored at -80°C for further use.

**Day 3. Transform with PCR-amplified knockout cassette.**

- 1) Inoculate 1 mL of the overnight culture into a 250 mL Erlenmeyer containing 90 mL low-salt LB, 9 mL of a 1 M L-arabinose solution, and hygromycin. Incubate at **30°C** with shaking or spinning.

Note: we placed the flask on top of a magnetic stir plate, and a magnetic stir bar inside the flask was spun to aerate the culture. The speed was kept at a slower rate (~500 rpm) than that used to transform pACBSR-Hyg or pFLP-hyg.

- 2) When the OD<sub>600</sub> reaches 0.4 – 0.6 (4 – 5 hours), make electrocompetent as above.
- 3) Electroporate 100  $\mu$ L of the electrocompetent cells with ~1  $\mu$ g of the FRT-flanked Apra<sup>R</sup> knockout cassette.
- 4) Add 500  $\mu$ L SOC to the cuvette, and transfer the mixture using a long gel-loading tip to a sterile 1.5 mL microfuge tube.
- 5) Incubate at 37°C with gentle shaking for 60 – 90 minutes.
- 6) Plate cells onto 2 LBApra plates and incubate overnight at 37°C.

**Day 4. A) Screen for transformants that have correct insertion of the knockout cassette.**

- 1) Pick 8 - 10 colonies and circle and number them. Dab each colony with a toothpick and suspend in 20  $\mu$ L water.
- 2) Incubate samples at **95°C** for 30 min.
- 3) Centrifuge the samples. Using the confirmation primers and the supernatant as the template, perform PCR to confirm correct insertion of the knockout cassette.

PCR Condition			
Cycles		Temp (°C)	Time (min)
1	Initial activation	95	15
	Denaturation	95	0.5
35	Annealing	52	0.5
	Extension	72	1.5 - 2
1	Final Extension	72	10
1	Post-PCR	4	$\infty$

- 4) Run the PCR products on an agarose gel. A correct knockout will have a size of about 1.2 to 1.3 kb (the approximate size of Apra<sup>R</sup> and flanking FRT sites) plus the length of DNA from the two ends of the deleted gene to the confirmation primers.

**B) Streak correct transformants for single colonies on LBApra at 37°C overnight.**

**Days 5 - 7. Pick 12 – 15 single colonies of the confirmed mutant and streak onto LBApra plates for 3 days at 37°C.**

**Day 8. Screen for loss of pACBSR-Hyg by streaking onto LBApra and low salt LB + hygromycin plates at 37°C overnight.**

- 1) Streak onto low salt LB + hygromycin and LBApra plates. Incubate at 37°C overnight.
- 2) If no hygromycin sensitive patch appears, pass an addition 2-3 days on LBApra and retest.

**Part II. Removal of resistance marker**

**Day 9. Culture mutant in 3 mL LBApra at 37°C overnight.**

- 1) If a hygromycin sensitive patch appears, inoculate it into 3 mL LBApra broth at 37°C overnight.

#### **Day 10. Transform with pFLP-hyg.**

- 1) Inoculate 1 mL of the overnight culture into a 250 mL Erlenmeyer containing 100 mL LB and apramycin. Incubate at 37°C with shaking or spinning.

Note: we placed the flask on top of a magnetic stir plate, and a magnetic stir bar inside the flask was spun to aerate the culture. The speed was approximately 800 – 1000 rpm.

- 2) When the OD<sub>600</sub> reaches 0.4 – 0.6 (~2.5 hours), make electrocompetent as above.
- 3) Electroporate 50 µL of the electrocompetent cells with 200 – 400 ng of pFLP-hyg.
- 4) Add 250 µL SOC to the cuvette, and transfer the mixture using a long gel-loading tip to a sterile 1.5 mL microfuge tube.
- 5) Incubate at **30°C** with gentle shaking for 60 minutes.
- 6) Spread 100 µL of the culture onto a low salt LB + hygromycin plate and incubate at **30°C** overnight.

#### **Day 11. Heat-shock mutants at 43°C overnight.**

- 1) Streak onto an LB plate for single colonies. Incubate at **43°C** overnight.

#### **Day 12. Screen colonies for loss of apramycin resistance.**

- 1) Use sterile toothpicks to pick 12 – 15 colonies and streak onto LB and LBApra plates at 37°C overnight.

#### **Days 13 – 15. Pass apramycin-sensitive mutants on LB plates 3 times.**

#### **Day 16. Screen for loss of pFLP-hyg by streaking onto LB and low salt LB + hygromycin plates at 37°C overnight.**

- 1) If no hygromycin sensitive streak appears, pass on LB plates for another 2-3 days and retest.
- 2) If a hygromycin sensitive streak appears, streak for single colonies on LB at 37°C overnight.

#### **Day 17. Confirm gene deletion by PCR and sequencing.**

- 1) Inoculate a single colony of the hygromycin-sensitive mutant into 3 mL LB and incubate at 37°C overnight with shaking.

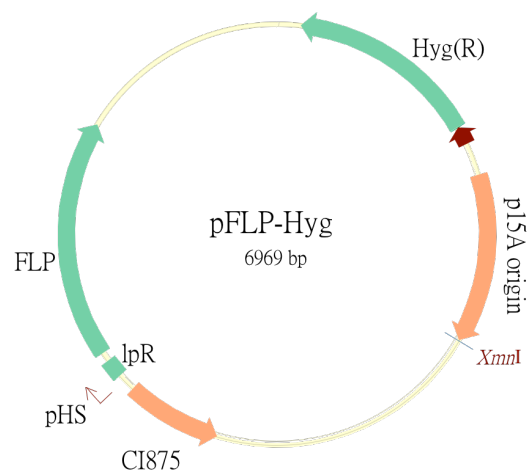
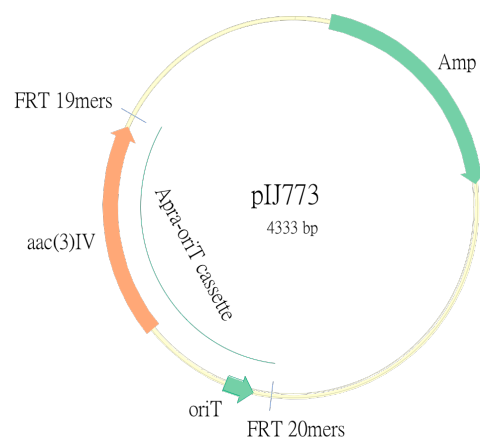
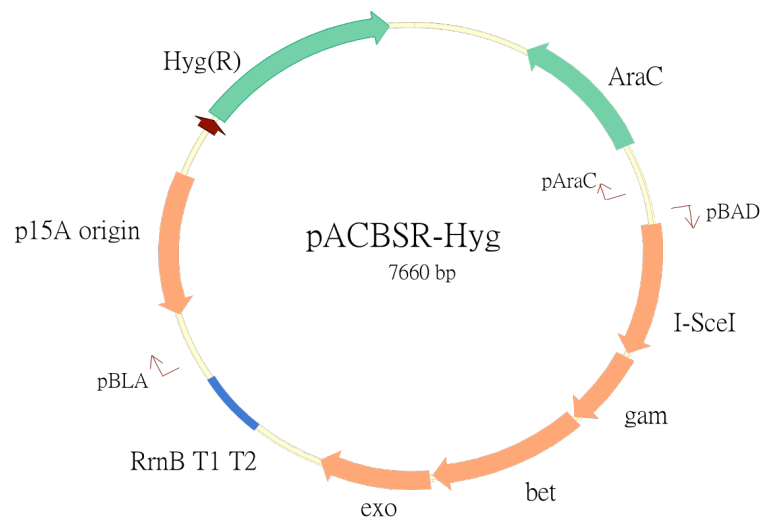


- 2) Using the same colony, suspend a small amount in ~20  $\mu$ L water.
- 3) Incubate the suspension at 95°C for 30 minutes.
- 4) Centrifuge the sample and use the supernatant as a template for PCR. Use the confirmation primers. Purify the PCR product and send it for Sanger sequencing. There should be a 81 bp scar region in place of the target gene.

**Day 18. Store strain at -80°C in 20% glycerol.**

## Appendix

### Plasmids



## **Primer design**

The first step of the knockout procedure is to design primers that target the apramycin selection marker and flanking FRT sites to the chromosomal location of interest. Approximately 60 bp of homology is sufficient. A set of confirmation primers is also needed to confirm that the knockout cassette has been inserted into the correct position in the chromosome and also to PCR and sequence the scar region once the apramycin resistance gene has been removed.

The template for PCR amplification of the apramycin selection marker and FRT sites is pIJ773. The homology arms can range from 39 to 80 bp, with 60 bp being the most common length.

- 1) *Forward knockout primer.* Locate the start codon for the gene of interest, then count 57 bp in the 5' direction to obtain a 60 bp homology region. Next, add the 'FRT20' sequence to the 3' end.

FRT20: 5' - attccggggatccgtcgacc - 3'

The forward primer, called the 'Forward KO primer (XXXX\_KO\_Fwd),' will therefore have the following design: 5' – 60 bp upstream homology – FRT20 – 3'

- 2) *Reverse knockout primer.* Locate the stop codon for the gene of interest, then count 57 in the 3' direction to obtain the other 60 bp homology region. Take the reverse complement of this sequence, then add the 'FRT19' sequence to the 3' end.

FRT19: 5' - ttaggctggagctgcttc - 3'

The reverse primer, called the 'Reverse KO primer (XXXX\_KO\_Rev),' will therefore have the following design: 5' – reverse comp of 60 bp downstream homology – FRT19 – 3'

### **Notes:**

- a. Always read the gene/operon in the direction of transcription so that the first three bases are the start codon (e.g., 'ATG'). This ensures that the knockout cassette is placed in the same orientation as the gene/operon it will replace.

## KO Primer Design Example

*zwf* gene in *K. pneumoniae* MGH 78578 (KPN\_02367), complement (2,593,162 .. 2,591,114):

5'-

CAATGGAAACGATTTTCATTAATGGGTGAAATCATTGGGCGTTTTTA **GCAAGAATATACCCCTCCG**CAAGCGCAAGCGG  
TGAGGAATGACGTGAAATGATGTTGTTTTTTTTTTCATTACATGATCAGGGTCGTATTTTACTGACCTGAACCAGGTAC  
AAACAACGAACAATAACGGCCGAATGCCCAAATCATCGCCGAATGTTTTACCCCTGTGCGGTTTTACCGCCGCAAAC  
GAAGGGGAAGGCTTTTCGGGAAATACGTAAACACAGTACAGTGCAGTGTAAAGAAAATTACAAATATAGCCTGGCAAAA  
**GCACCAGACTATCAACTGAGGAGAATGACATG**CGCGGTAAACGAAACGGCCAGGCATGCGACCTGGTCATTTTCGGC  
GCGAAGGGCGACCTGGCGCGGCGTAAATTGTTGCCTTCCCTGTATCAGCTTGAAAAGGCGGGCCAGATCCACGCTGA  
CACCAGAATCATTGGCGTCGGCCGTGCCGACTGGGATAAAGCGGCTTATACCAAAGTCGTGCGCGAAGCGCTGGAAA  
CCTTCATGAAGGAAAAAATTGATGAAGGTTTGTGGGATACCTGAGCGGACGCCTGGAGTTCTGTAATCTTGACGTC  
AATGACACCAGCGGCTTCACCCGCCTGGGTGAGATGCTGGATCAAAGAACCCTGTCACCATCAACTATTTTCGCTAT  
GCCGCCGAGCACCTTTGGCGCGATCTGTAAAGGGCTGGGCGAAGCGAAGCTTAATGCCAAGCCTGCGCGCGTGGTCA  
TGGAAAAACCGCTCGGCACCTCGCTGGAAACCTCCCGTGAAATCAACGACCAGGTGCGCGAGTTTTTTGAAGAGTGT  
CAGGTCTACCGTATCGACCACTATCTCGGTAAAGAGACGGTCCTCAACCTGCTGGCGCTGCGCTTCGCCAACTCCCT  
GTTTCGTCAATAACTGGGACTGCCGCACCATCGATCACGTGAAATCACCGTCGCGGAAGAGGTGGGTATTGAAGGGC  
GCTGGGGTTACTTCGACCAGGCCGGGCAGATGCGCGACATGATCCAGAACCACCTGCTGCAGATCCTGTGCATGATT  
GCCATGTACCGCCGTCCGATCTCAGCGCCGACAGCATCCGTGATGAAAAGGTCAAGGTGCTGAAATCCCTGCGCCG  
TATTGACCGCTCCAACGTGCGCGAGAAAACCGTTCGCGGCCAGTATACCGCCGGCTTTGCCAGGGCAAAAAAGTGC  
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TTACTTTCAAACCCCGGAAGTGAATCTGTTTAAAGAGACCTGGCAGGAGCTGCCGAGAACAAGCTGACCATTCGCC  
TGCAGCCGGACGAAGGGGTAGATATCCAGGTACTGAACAAAGTGCCGGGCCTCGATCATAAACATAACCTGCAGATC  
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GACCATGCGTGGCATCCAGGCGCTGTTCTGTTCCGCCGACGAAGTAGAAGAGGCGTGGAATGGGTTGACTCCATTA  
CCGAAGCCTGGGCAGCCGACCGCGATGCGCCGAAACCGTATCAGGCCGGGACCTGGGGACCGGTTGCGTCGGTGGCC  
ATGATCACTCGCGACGGTCGTTTCATGGAACGAGTTCGAG **TAA**GGCTGGAGGCTAATCCGTCAGGGTTATTTTACCGG  
**TAACATGATCTGACGCAGAAAGT**CGCACGCTTTTTTAATCTTTTAAGCCCCGTGAGGATTCACCCACGGGGCTTTTTT  
TATTAACTGCCTGCAGACATTTTGGCCCTGAGCGCTGGTGTGTTGTGGCCTTTACCACGTCAACACGTCATCA **CGCA**  
**GCGGCCGGACAG**ATAAAAATTTGAGGAGCCTTTATGAATTCGACA-3'

*zwf*\_KO\_Fwd

5'-AAGAAAATTACAAATATAGCCTGGCAAAAGCACCAGACTATCAACTGAGGAGAATGAC**ATG**attccgggatccgtcgacc-3'

*zwf*\_KO\_Rev

5'-ACTTTCTGCGTCAGATCATGTTACCGGTAAAATAACCTGACGGATTAGCCTCCAGCC**TTA**tgtaggctggagctgcttc-3'

*zwf*\_Conf\_Fwd

5'-**GCAAGAATATACCCCTCCG**-3'

*zwf*\_Conf\_Rev

5'-**CTGTCCGGCCGTCGCG**-3'

## References

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(<http://www.pnas.org/content/100/4/1541.abstract>)